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MOLECULAR PHARMACOLOGY, 29:7-15

Expression of β-Adrenergic Receptors in Synchronous and Asynchronous S49 Lymphoma Cells. I. Receptor Metabolism after Irreversible Blockade of Receptors and in Cells Traversing the Cell Division Cycle

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Received August 5, 1985; Accepted October 10, 1985

SUMMARY

We have examined the metabolism of β-adrenergic receptors in intact S49 lymphoma cells. Centrifugal elutriation was used to prepare synchronized cells enriched in particular phases of the cell division cycle. In these synchronized cells, the rate of appearance of β-adrenergic receptors [i.e., [^{125}I]iodocyanopindolol binding sites) was continuous, approximately 75 sites/cell/hr, and receptor number per cell increased in proportion to the increase in cell size. Thus, receptor number, normalized for cell size, remains constant throughout the cell cycle. Receptors on cells in G_1, S, and G_2/M phases of the cell cycle displayed similar affinities for the radiolabeled antagonist [^{125}I]iodocyanopindolol and apparent affinities for the agonist isoproterenol. We examined rates of receptor turnover in asynchronous cells by following receptor recovery after inactivation of β-adrenergic receptors with bromoacetylalprenololmenthane (BAAM), an irreversible β-adrenergic antagonist. The β-adrenergic receptors on S49 cells demonstrated an average "half-life" of 28–30 hr. Since the population doubling time for S49 cells is 16–17 hr, this would indicate that receptor protein is conserved through successive cell generations. Moreover, receptor reappearance after blockade by BAAM was a function of newly appearing receptors during the S49 cell cycle and not loss of BAAM from receptors. The rate of receptor metabolism indicates that, under basal conditions (i.e., in the absence of agonist), β-adrenergic receptors on S49 cells are metabolized more slowly than are other classes of receptors that bind peptides and cholinergic agonists in several other cell types.

The interaction between an agonist and its receptor on an intact cell results not only in the generation of primary responses, such as ion movement or cAMP generation, but can also lead to alterations in receptor response (desensitization) and receptor number (down-regulation) (1–5). One important factor which contributes to the regulation of receptors by agonists and other perturbants is the expression of receptors in the "basal state," i.e., in the absence of exogenous agonist. In the present studies, we set out to define properties of basal metabolism of β_2-adrenergic receptors in the S49 lymphoma cell line, a widely used model system for studying β-adrenergic receptors and adenylate cyclase (6, 7). For our studies, we employed two independent approaches: 1) analysis of the expression of receptors as S49 cells traverse the cell cycle, and 2) irreversible blockade of β-adrenergic receptors and analysis of the subsequent recovery of receptors to basal "steady state" levels.

In the latter set of experiments, we inactivated β-adrenergic receptors on intact S49 cells by incubation of cells with the irreversible β-adrenergic antagonist BAAM (8). BAAM undergoes covalent attachment to receptor protein through bromoalkylation of sulfhydryl groups and thus irreversible occupancy of the receptor binding site. We compared the values obtained for receptor appearance and turnover in asynchronous cultures of S49 cells to properties of receptor expression in synchronized cultures of S49 cells. Cell cycle-synchronized populations of

This work was supported by Grants 82-07498 and 85-02168 from the National Science Foundation. L. C. M. was partially supported as a predoctoral trainee on a Training Grant in Hypertension from the National Institutes of Health (HL 07261).

ABBREVIATIONS: BAAM, bromoacetylalprenololmenthane; DME/HS, Dulbecco's modified Eagle's medium containing 10% heat-inactivated horse serum; DME/H/BSA, Dulbecco's modified Eagle's medium containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid in lieu of $NaHCO_3$ and 1 mg/ml bovine serum albumin; ICYP, iodocyanopindolol; CGP, CGP-12177 or 4-(3-*tert*-butylamino-2-hydroxypropoxy)benzimidazole-2-on hydrochloride.

S49 cells were obtained by centrifugal elutriation, a noninvasive (i.e., without using drugs or other perturbants, like double thymidine block) means to isolate these populations of S49 cells (9). Data obtained with cells that were synchronized aided in the interpretation of results of receptor turnover in asynchronous, continuously growing cells.

Materials and Methods

Cell lines and cell culture. Wild-type (24.3.2) S49 lymphoma cells were grown at 37° in suspension culture in DME/HS in a 10% CO_2:90% air environment. Cells were used for experiments only when growing logarithmically (i.e., $<1 \times 10^6$ cells/ml) and when viability was >90% as assessed by trypan blue (0.05%) exclusion. Cell counts were monitored on a Coulter ZBI cell counter/Channelyzer. In previous studies we have determined that heat-inactivated horse serum contains a very low (<1 nM) concentration of norepinephrine and virtually no epinephrine (10). Thus, the media used in these studies expose cells to negligible levels of agonist.

β-Adrenergic receptor binding assay. Cells in DME/HS were centrifuged at 300 × *g* for 5 min at room temperature and resuspended in DME/H/BSA. Receptor number on intact cells was measured using the radiolabeled β-adrenergic antagonists [^{125}I]ICYP or [^{3}H]CGP as previously described (11, 12). Typically, replicates (4–6) containing 0.5–1 × 10^6 cells (for [^{125}I]ICYP) or 3–5 × 10^6 cells (for [^{3}H]CGP) were incubated in a shaking water bath at 37° with radioligand in the absence (total binding) or presence of 1 μM (−)-propranolol (nonspecific binding) in a final volume of 0.5 ml for 60 min. Binding was terminated by dilution (20-fold) and rapid filtration over Whatman GF/C filters, followed by washing with 20 ml of either 5 mM potassium phosphate, pH 7.0, 1 mM $MgSO_4$, or phosphate-buffered saline. Filters were counted in a gamma counter (for [^{125}I]ICYP) at 80% efficiency or in a liquid scintillation counter (for [^{3}H]CGP) at 46% efficiency. [^{125}I]ICYP was iodinated and prepared as previously described (11). The equilibrium dissociation constant (K_D) and the maximal number of binding sites (B_{max}) were determined by Scatchard analysis of saturation binding isotherms. Data for competition of (−)-isoproterenol for [^{125}I]ICYP binding sites were analyzed on a computer using LIGAND, a nonlinear least squares regression program (13).

Receptor inactivation protocols. BAAM was made up fresh before each experiment in 100% ethanol. BAAM was added to cells in growth conditions (DME/HS, 37°) at a 1:1000 or greater dilution (≤0.1% ethanol) to achieve the desired concentration. Ethanol alone was added to control cultures of cells. Cells were typically in log phase growth (0.5–1 × 10^6 cells/ml). At the end of the desired incubation period, cells were centrifuged at 300 × *g* and washed an additional three to four times by resuspension and centrifugation in DME/H/BSA, pH 7.4, containing 10% horse serum.

Centrifugal elutriation of S49 cells. Asynchronous cultures of wild-type S49 cells in log phase growth ($<1 \times 10^6$ cells/ml) were loaded into the separation chamber of a Beckman JE-6 elutriator rotor according to previously described procedures (9). Rotor speed was generally 1900–2000 rpm (~400 × *g*) and elutriations were performed at a rotor temperature of 22° with 37° elutriation medium (DME/H/BSA + 5–10% horse serum). Typically, 3.5–4.5 × 10^8 cells in 20–30 ml of sterile DME/H/BSA (37°) containing 5–10% horse serum were introduced into the chamber at an initial flow rate of <12 ml/min until cells had aligned according to size (generally 10 min). Cells were eluted from the rotor by incremental increases in flow rate to obtain the desired cell cycle-specific fraction. Two protocols were used. In the first protocol, cells were loaded into the elutriation rotor at < 12 ml/min and an early eluting fraction of cells (12.5–13.5 ml/min at 400 × *g*) was obtained; this fraction represented approximately 10–14% of the starting population of cells. At higher flow rates, additional fractions from the middle range of distribution of cell volume (18–19.5 ml/min) and those cells with the largest volume (>21 ml/min) were collected. Cytofluorometric analysis of the DNA content of fixed cells from these fractions revealed that cells with the smallest volumes (the early eluting fraction) were exclusively G_1 cells, cells obtained from the mid-volume range were predominantly in S phase, and cells with the largest volumes were primarily in the G_2 phase of the cell cycle but contained mitotic cells as well and were thus termed G_2/M cells. Thus, studies carried out on three fractions of cells enriched in G_1, S, or G_2/M phases of the cell cycle are "cross-sectional" analyses. In the second protocol only homogeneous G_1 cells were obtained and then returned to sterile culture to progress synchronously through their cell cycle; these we term "longitudinal" studies. During elutriation, we monitored isolation of cells by measuring average distributions of cell volumes with a Coulter counter/Channelyzer that had been calibrated with 9.90-μm-diameter microspheres.

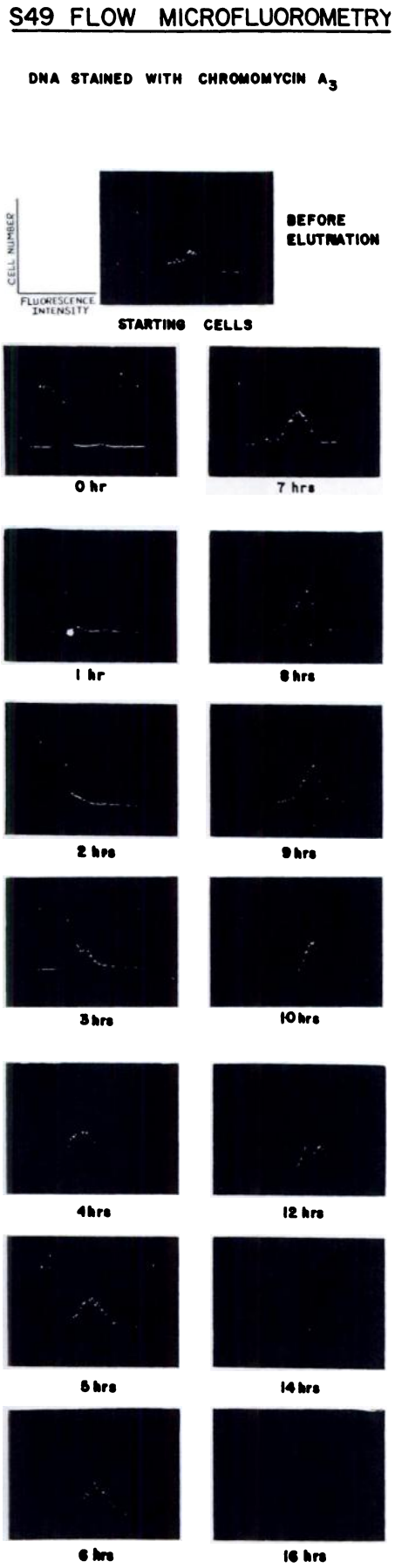
Determination of cell cycle phase distribution. Analysis of total cellular DNA content to determine cell cycle phase position in elutriated fractions was performed by flow cytofluorometric analyses. Aliquots of elutriated cells were fixed in a final concentration of 35% ethanol in phosphate-buffered saline and DNA stained by Chromomycin A_3 (14). DNA histograms were obtained from sampling ≥ 10,000 cells per fraction. Analysis was performed on a fluorescence activated cell sorter (designed and built at the University of California, San Diego, by Dr. Siddhartha Sarkar) as described previously (15). The computer program used to analyze cycle-specific fractions is similar to ones originally reported by groups at Los Alamos and Lawrence Livermore Laboratories (16, 17).

Materials. BAAM was the generous gift of Dr. Joseph Pitha, Gerontology Research Center, the Francis Scott Key Medical Center (Baltimore, MD). (±)-Cyanopindolol (Dr. G. Engel, Sandoz Ltd.), (−)-isoproterenol (Sterling-Winthrop), and (−)-propranolol (Ayerst) were also gifts. All other reagents were of the highest quality and were obtained from standard sources.

Results

Separation of wild-type S49 cells into synchronized cell populations by centrifugal elutriation. S49 cells were separated into cell cycle-specific fractions by the process of centrifugal (or counterflow) elutriation, which allows one to isolate cells primarily on the basis of cell volume and, to a lesser extent, cell density and shape (18–20). As described in Materials and Methods, we performed both cross-sectional and longitudinal studies to assess expression of β-receptors in S49 cells. A representative cytofluorometric analysis of a longitudinal progression of S49 cells through the cell cycle is shown in Fig. 1. An additional approach that aided both in the fractionation of asynchronous cells and in the assignment of cell cycle position was measurement of cell volume. We found that estimates of cell volume correlated closely with cytofluorometric data from fixed cells. Thus, in a typical longitudinal study, synchronous G_1 cells showed a progressive increase in volume until mitosis, after which new daughter cells appeared in G_1' (the new G_1 phase; see Fig. 2, *top panel*). The number of synchronized cells remained constant, until approximately 10 hr, after which mitosis occurred; a doubling of cell number was observed by 16–17 hr (Fig. 2, *bottom panel*), which is in excellent agreement with the doubling times obtained in asynchronous S49 cells (Refs. 9 and 17, and below).

Expression and properties of β-adrenergic receptors in synchronized S49 cells. [^{125}I]ICYP binding progressively increased from ~700 sites/cell in G_1 cells to ~1400 sites/cell as cells approached mitosis (Fig. 3). Binding sites then fell approximately to G_1 levels in the newly appearing daughter (G_1') cells. When the rate of receptor expression from G_1 to mitosis was analyzed by linear regression (Fig. 4, *left*), a value of 75 sites/cell/hr ($r^2 = 0.88$) was obtained. In addition, if receptor expression was corrected for cell size (μm^3) at each time point (Fig. 4, *right*), we found a fixed relationship between receptor



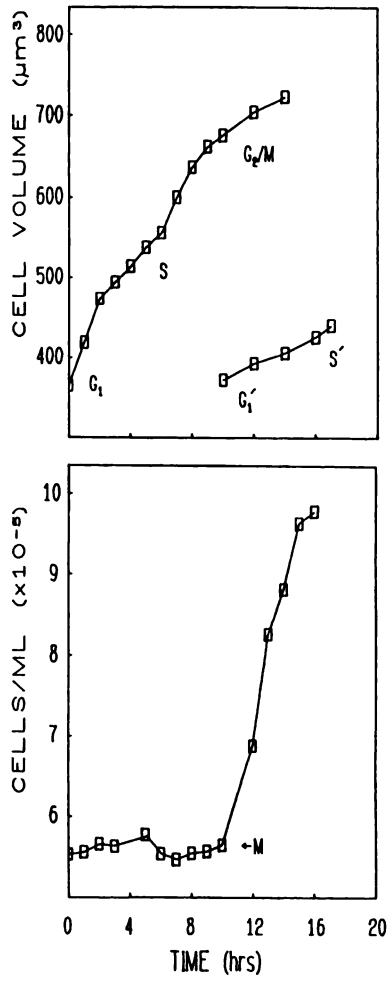


Fig. 2. Cell size and population kinetics in synchronized S49 cells. G_1 cells obtained by elutriation were returned to culture and, at the times indicated, aliquots of cells were taken for the determination of cell volume (*top*) or culture density (*bottom*). Cell volume was measured in a Coulter cell counter/Channelyzer standardized with 9.90-μm-diameter calibration microspheres. Initial seeding density was 0.5×10^6 cells/ml. Major cell cycle positions are indicated. In the population density study (*bottom*), *M* denotes the earliest time of detectable daughter cells (mitosis). Data shown are identical to those obtained in seven additional experiments.

number and cell size during the cell cycle.[1] Values for cells undergoing mitosis were omitted due to the mixture of both M and G_1' cells. Similar results were obtained using the β-adrenergic antagonist [^{125}I]iodohydroxybenzylpindolol in another longitudinal study (Fig. 4, *right*). Moreover, the increase in [^{125}I]ICYP binding as cells progressed through G_1, S, and G_2/M was the consequence of an increase in receptor number without a change in the affinity of the receptors for [^{125}I]ICYP

[1] Cell volume was calculated assuming that S49 cells are approximately spherical and that increases in cell size are a consequence of increases in cell diameter and not of expression of extensive microvillus projections. Scanning electron microscopy of fixed S49 cell fractions enriched for G_1, S, and G_2/M phase cells supported this assumption (data not shown).

Fig. 1. Cytofluorometric analysis of DNA content in synchronized S49 cells during the transition through the cell cycle. G_1 cells ($3–6 \times 10^7$) obtained by elutriation were returned to culture resuspended in fresh growth medium. At the times indicated, aliquots of cells were taken and stained for DNA content with chromomycin A_3 in order to determine position within the cell cycle by cytofluorometric analysis. Data shown are similar to those obtained in three separate experiments.

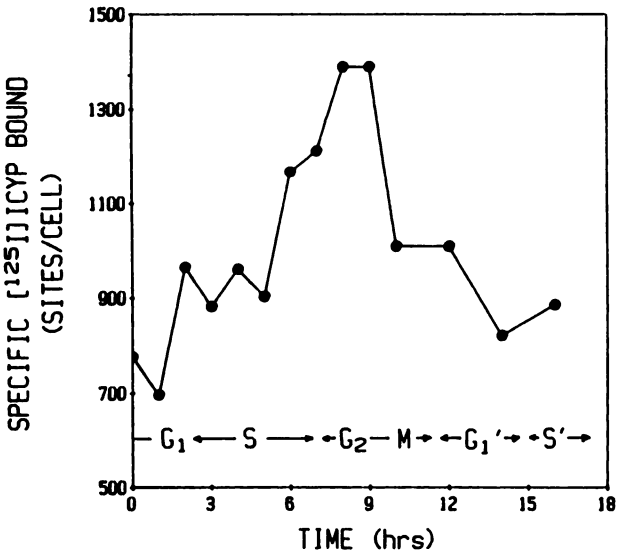


Fig. 3. Cell cycle-dependent expression of β-adrenergic receptors in synchronized S49 cells. G_1 cells obtained by elutriation were returned to culture. At the times indicated, aliquots of cells (0.5×10^6) were assayed in triplicate for specific binding of [^{125}I]ICYP (100 pM) as described in Materials and Methods. Binding has been corrected for cell number and is expressed as sites/cell. Population density measurements and cytofluorometric analysis indicated that >95% of the G_1 cells traversed the cell cycle. Cell cycle positions are indicated at the *bottom*. Similar results were obtained in a separate longitudinal experiment using [^{125}I]iodohydroxybenzylpindolol. These data are shown in Fig. 4.

(Table 1). Thus, β-adrenergic receptor number increases as a function of cell size (volume) and progression through the S49 cell cycle.

The receptors that appeared during a single traverse of the cell cycle had a similar apparent affinity for the agonist isoproterenol (Table 1). Competition by isoproterenol for [^{125}I]ICYP binding sites on intact cells was complex (pseudo-Hill coefficient <1). Two populations of sites with different affinities were observed, and these two populations could be resolved by analysis of the binding data using LIGAND (13). Little difference was observed among G_1, S, or G_2/M cells in either the proportions of high affinity and low affinity binding sites (20% and 80%) or their affinities for isoproterenol (~10 nM and ~3 μM, respectively). These values are identical to those obtained with asynchronous cells (11).

Blockade of β-adrenergic receptors on S49 wild-type cells by BAAM. Without independent estimates of rates of receptor appearance and disappearance, one cannot presume that expression of β-adrenergic receptors in synchronized populations of S49 cells necessarily reflects the rate of receptor appearance alone. If receptor turnover is sufficiently slow in these cells, then the expression of β-receptors in synchronized cells may be a measure of the rate of *de novo* receptor appearance in the absence of significant degradation of newly appearing receptors. In order to obtain an independent estimate of β-receptor turnover in S49 cells, we blocked β-receptors on intact asynchronous cells with the irreversible β-adrenergic antago-

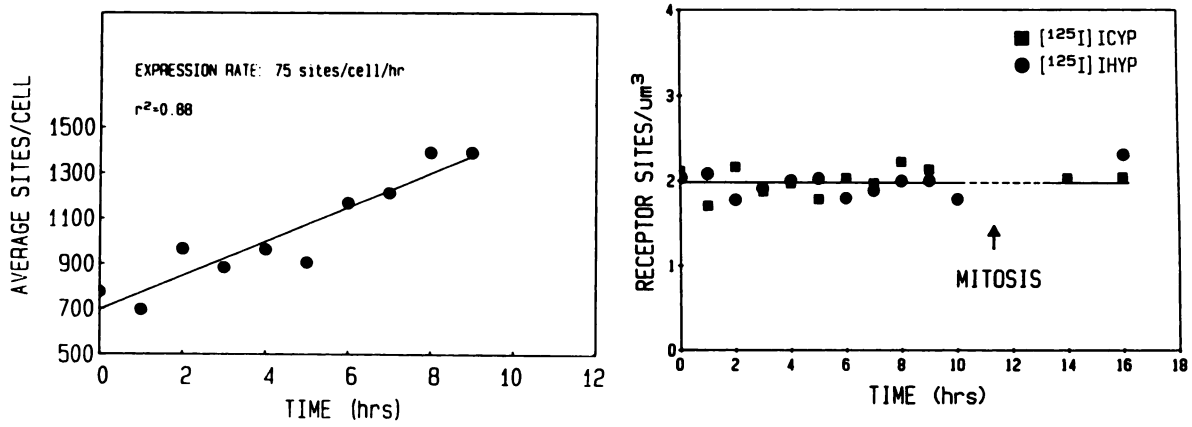


Fig. 4. Rate of receptor expression and relationship of β-adrenergic receptor number to cell size during the S49 cell cycle. *Left*, Rate of receptor expression. Data for the expression of [^{125}I]ICYP during the S49 cell cycle in Fig. 3 were analyzed by linear regression. Only data from G_1 until mitosis were used in order to omit mixed populations of cells during mitosis. Rate of receptor expression was equal to ~75 sites/cell/hr ($r^2 = 0.88$). *Right*, Relationship of receptor number to cell size. Data from the experiment for [^{125}I]ICYP (Fig. 3) and from a similar study using [^{125}I]IHYP to measure the expression of β-adrenergic receptors during the cell cycle were normalized for cell size (μm³). Data points during mitosis (– – –) were omitted due to mixed cell populations (M and G_1', the subsequent G_1 period).

TABLE 1
β-Adrenergic receptor characteristics in G_1, S, and G_2/M S49 cells

	G_1	S	G_2/M
Scatchard Analysis[a] (N = 3)			
K_D (pM)	14 ± 1.7	17 ± 2.9	13 ± 0.3
B_{max} (sites/cell)	970 ± 125	1159 ± 105	1553 ± 248
Isoproterenol Competition[b] (N = 4)			
K_{high} (nM)	8.9 ± 5.6	12 ± 6.0	5.9 ± 2.3
% Sites	19 ± 3.4	21 ± 2.5	17 ± 4.7
K_{low} (μM)	2.8 ± 0.5	3.3 ± 0.6	3.2 ± 0.6
% Sites	81 ± 3.4	79 ± 2.5	84 ± 4.7

[a] Values were obtained from analysis of [^{125}I]ICYP saturation binding isotherms at 37°.
[b] Values were obtained from LIGAND analysis. All two-site fits were significant ($p < 0.02$).

nist, BAAM, and then performed kinetic analyses of the recovery of receptors to the original steady state level of receptor expression.

Initial studies were performed to establish conditions in which BAAM could be used to inactivate β-receptors on intact S49 cells. Preliminary experiments indicated that BAAM could be used to block receptors on cells under growth conditions so as to minimally perturb the cellular environment. Blockade of receptor binding sites appeared irreversible, withstanding numerous washes, and could be inhibited when coincubated with the reversible β-adrenergic antagonist propranolol. Protection from BAAM by (−)-propranolol occurred at about 100-fold lower concentrations than by (+)-propranolol (data not shown). Two general protocols for the use of BAAM were used: 1) an 18-hr (overnight) incubation with cells to achieve maximum blockade, and 2) shorter (0.5–1.0 hr) incubations to achieve variable degrees of receptor inactivation.

An overnight incubation (18 hr) of S49 cells with low (≤1.0 μM) concentrations of BAAM in growth medium blocked approximately 65% of β-receptors with no change in cell viability (Fig. 5, *top*). As would be expected for the irreversible alkylation of receptors, a number of BAAM concentrations showed similar degrees of receptor blockade from this long incubation. Assessment of remaining receptors by saturation binding isotherms of [^{125}I]ICYP indicated that the loss in binding produced by treatment with BAAM reflected a loss of receptor number (control: 1540 sites/cell; treated: 480–530 sites/cell) and not competitive blockade of receptors by residual BAAM. Cells treated with several different concentrations of BAAM had similar K_D values for [^{125}I]ICYP: control (21 pM), 0.1 μM BAAM (16 pM), 0.3 μM BAAM (23 pM), and 1.0 μM BAAM (36 pM). In order to block an even greater number of receptors, we treated cells that had been incubated overnight with 0.1 μM BAAM with a short (1 hr) incubation with 1.0 μM BAAM. This protocol decreased the number of [^{125}I]ICYP sites by 80% (Fig. 5, *bottom*), but slightly increased the K_D for [^{125}I]ICYP, suggesting that some residual BAAM may have remained after cells were washed to remove the drug. Accurate estimates of K_D in these experiments, however, were hampered by the low ratio of specific to nonspecific binding of [^{125}I]ICYP. Treatment of cells with 1 μM BAAM increased the K_D for [^{125}I]ICYP in some studies; thus, we extensively washed S49 cells treated with BAAM in all subsequent experiments and performed binding studies with maximally saturating concentrations of [^{125}I]ICYP.

We have recently described the existence of an intracellular pool of β-adrenergic receptors in S49 cells that represents about 15% of the total cellular receptor number (21). This pool can be detected at 37° by the differential accessibility of membrane-permeant ([^{125}I]ICYP, [^{3}H]dihydroalprenolol) but not membrane-impermeant ([^{3}H]CGP) β-adrenergic antagonists and the susceptibility of these bound ligands to elution from the cell surface by low pH (21). When β-receptors on intact S49 cells were blocked to varying degrees with BAAM at 37°, followed by assessment of remaining receptors by either [^{125}I]ICYP (total receptors) or [^{3}H]CGP (surface receptors), identical levels of remaining receptors were identified (Fig. 6) relative to control, untreated cells. This implies that BAAM, which is a hydrophobic molecule, was able to block surface as well as intracellular receptors at 37°. Thus, measurements of receptors reappearing after receptor inactivation by BAAM (to be described below) probably represent identification of the intracellular receptors capable of binding [^{125}I]ICYP as well as those receptors appearing on the cell surface.

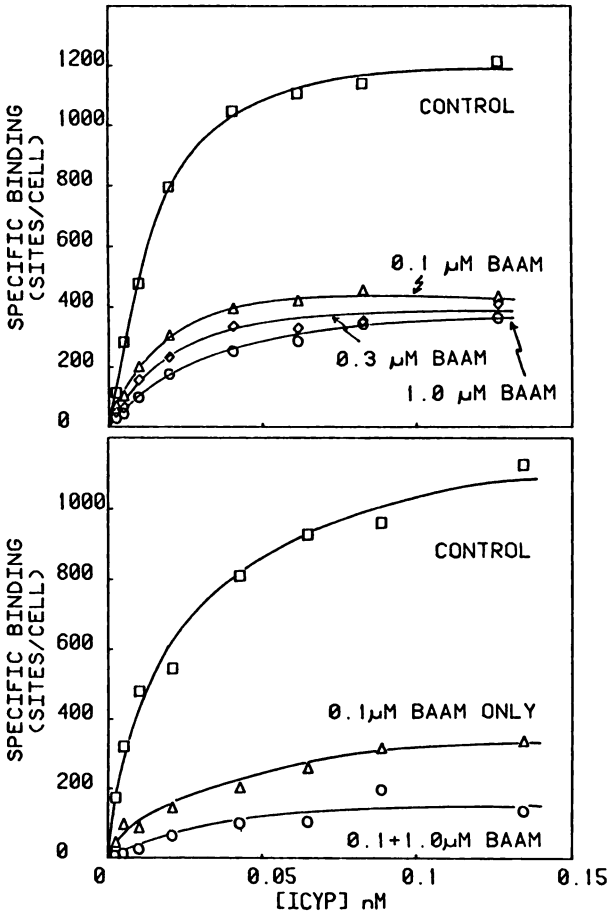


Fig. 5. Saturation binding isotherms of [^{125}I]ICYP in wild-type S49 cells after treatment with BAAM. *Top*, Cells (0.5–1.0 × 10^6) were treated with the indicated concentrations of BAAM in growth medium (DME/HS) for 18 hr at 37°. Cells were then washed four times with DME/H/BSA containing 10% horse serum and resuspended in DME/H/BSA. Aliquots (0.7 × 10^6 cells) were incubated with the indicated concentrations of [^{125}I]ICYP for 1 hr at 37°. Nonspecific binding was determined by 1.0 μM (−)-propranolol. Control cells (□——□) had a K_d = 21 pM and a B_{max} = 1543 sites/cell. Cells treated with 0.1 μM (Δ——Δ), 0.3 μM (◇——◇), and 1.0 μM (○——○) BAAM had K_D:B_{max} values of 16 pM:532 sites/cell, 23 pM:488 sites/cell, and 36 pM:487 sites/cell, respectively. *Bottom*, Cells were treated identically as above except that one group of cells treated with 0.1 μM BAAM for 16 hr was incubated with an additional 1.0 μM BAAM and allowed to incubate one additional hour. Control cells (□——□) had K_D:B_{max} values of 12 pM:1104 sites/cell. Cells treated with 0.1 μM BAAM alone (Δ——Δ) had values of 18 pM:351 sites/cell. Cells treated with two doses of BAAM (○——○) had values of 75 pM:215 sites/cell.

β-Adrenergic receptor turnover in wild-type S49 cell after receptor inactivation by BAAM. Asynchronously growing S49 cells were treated with BAAM and returned to log phase culture; then, at various time intervals, aliquots of cells were assayed for cell growth (Fig. 7, *top*) and specific binding of [^{125}I]ICYP (Fig. 7, *bottom*). Cells treated with BAAM were >95% viable and had no significant alteration in average doubling time (16.6 hr) compared to control cells (17.0 hr). Receptor reappearance began immediately and progressed in a continuous manner. Treated cells approached an average steady state level of receptor expression nearly identical to that of control cells by approximately 100 hr.

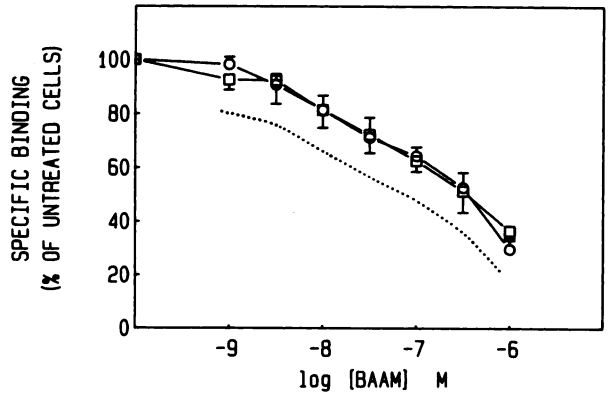


Fig. 6. Inhibition of [^{125}I]ICYP and [^{3}H]CGP binding to intact S49 cells after treatment with BAAM. Cells were incubated with the indicated concentration of BAAM in growth conditions for 60 min at 37°, washed extensively, then assayed for specific binding of [^{125}I]ICYP (100–120 pM) (□——□) or [^{3}H]CGP (2–5 nM) (○——○). Data shown are the means ± SE from four separate experiments for each radioligand. The *dotted line* represents the theoretical level of [^{3}H]CGP binding if BAAM blocked only surface receptors in S49 cells with a 15% intracellular receptor pool accessible to [^{125}I]ICYP.

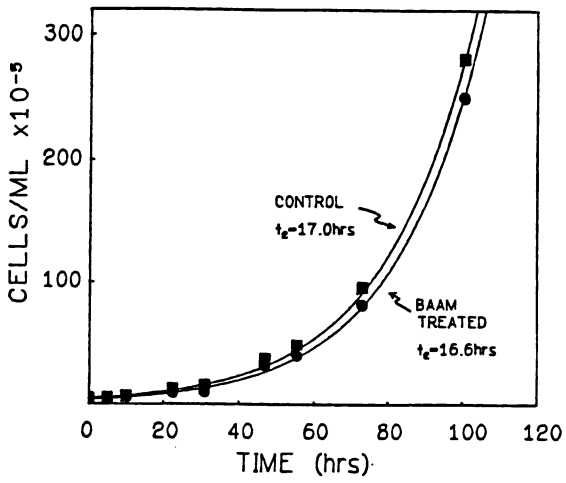
If production of receptors is constant over the entire period of receptor reappearance, and the degradation of receptors produced in treated cells is proportional to the concentration of these receptors on the cells, then receptor reappearance may be expressed as

$$[R_t] = k_a/k_d(1 - \exp^{k_d t}) + R_o\exp^{-k_d t} \qquad (1)$$

where k_a is the rate of receptor appearance, k_d is the rate constant of receptor disappearance, and R_o is the initial receptor concentration following treatment with BAAM (22, 23). At steady state ($t >>>> 0$), average receptor number (R_{ss}, in sites/cell) will reach that of control cells and will be equal to k_a/k_d. This has been shown to be true for a number of receptor systems, such as those for insulin (22), α_1-adrenergic agonists (23), acetylcholine (24), and epidermal growth factor (25).

Data for receptor reappearance shown in Fig. 7 were analyzed by nonlinear least squares regression according to the method of Marquardt (26), using Eq. 1. We obtained values of 32 sites/cell/hr for receptor reappearance and 0.023 hr^{-1} for the rate constant of receptor disappearance. As will be shown subsequently, this value for receptor reappearance is an underestimate of the "true" reappearance rate because one must also consider the contribution of cell growth in this setting. The disappearance rate constant represents an average half-life of ~30 hr for β-adrenergic receptors in asynchronous cultures of S49 cells. The calculated value of R_{ss} for BAAM-treated cells was 1390 sites/cell, in excellent agreement with the control average of 1410 sites/cell over the course of the experiment.

Values for the rate constant of receptor disappearance from this and two additional experiments yielded $k_d = 0.025 \pm 0.002$ hr^{-1} (mean ± SE, $n = 3$) and, therefore, a mean half-life of ~28 hr. Scatchard analysis of [^{125}I]ICYP binding at 48 and 72 hr indicated that recovery consisted of an increase in receptor number, not an alteration of the affinity of receptors for [^{125}I]ICYP (data not shown). In several different experiments, cells displayed a variable steady state number of receptors (650–1400 sites/cell), a phenomenon perhaps attributable to the presence of serum (9, 27) or to use of different batches of cells. Interestingly, the rate constants of receptor disappearance were very similar in these experiments, and variation in the appear-



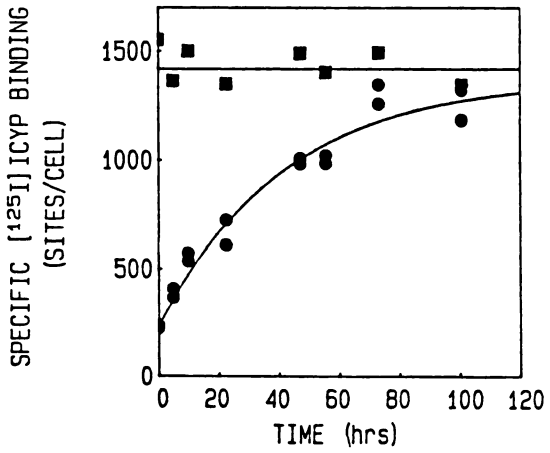


Fig. 7. S49 cell growth and receptor recovery following receptor inactivation by BAAM. *Top*, Cells (0.5–1.0 × 10^{6}) were treated with 0.1 μM BAAM (16 hr) followed by 1.0 μM BAAM in DME/HS (1 hr) as described in the text, washed, and diluted to 0.4 × 10^{6} cells/ml in DME/HS, and returned to the incubator. Cells were maintained for several days by daily dilution and replenishment with fresh DME/HS. At the times indicated, aliquots of cells were removed and assayed for cell density and viability with a Coulter cell counter/Channelyzer. The data shown are representative of those obtained in four separate experiments and are the mean of quadruplicate cell counts at each time point for control cells. Values for BAAM-treated cells are the mean of cell counts from duplicate, individually treated flasks. Cell counts were corrected for dilution of the culture. Doubling times were obtained by nonlinear least squares regression analysis of data on a computer that was fit to an exponential growth equation (Eq. 2). The mean doubling times (±SE) from four experiments (employing different BAAM incubations) were 17.0 ± 0.43 hr for control and 17.2 ± 0.56 hr for BAAM-treated cells ($r^2 > 0.99$). *Bottom*, β-Adrenergic receptor recovery in S49 cells following receptor inactivation by BAAM. Cells (0.5–1.0 × 10^{6}) were treated with BAAM, washed, and returned to sterile culture conditions as described. At the indicated times, cells were removed, centrifuged, and resuspended in DME/H/BSA. Aliquots (0.7–1.0 × 10^{6} cells) were incubated with 90–120 pM [^{125}I]ICYP and specific binding was determined. Data for the BAAM-treated cells are the mean values from six binding replicates for each of two individually treated flasks. These data were fit to the equation described in the text (Eq. 1). Values for k_a = 32 sites/cell/hr, and k_d = 0.023 ± 0.004 hr^{-1} (regression coefficient ± SE) were obtained by nonlinear least squares regression analysis using a value for R_0 = 231 sites/cell. The calculated receptor half-life was 30 hr.

ance rate of receptors accounted for the differences in steady state number of receptors.

In continuously growing cells, such as S49 cells, the rate of receptor appearance reflects production of receptors necessary to maintain a steady state number of receptors on what might be considered an "average" cell in a culture.[2] This "average" cell comprises cells at varying stages in the cell cycle. To assess the rate of receptor appearance for any particular cell in a culture of continuously dividing cells, one must incorporate the equation for cell growth:

$$C_{(t)} = C_o \exp^{k_c t} \quad (2)$$

where $C_{(t)}$ is the total number of cells (measured at time, t), C_o is the initial cell density, and k_c is the growth rate constant, into Eq. 1 for receptor production.

The resulting equation for receptor production in continuously dividing cells becomes

$$[R_t] = \frac{k_a C_o}{k_c + k_d} \exp^{k_c t} + \left(R_o - \frac{k_a C_o}{k_c + k_d}\right) \exp^{-k_d t} \quad (3)$$

where R_t is the total number of receptors produced in culture at time, t, after blockade of receptors by BAAM.[3] Using values for the above constants derived experimentally (see legend to Fig. 8), we asked whether the estimate of rate of change of receptor expression during the cell cycle could be used as the value for k_a, the rate of receptor appearance, and could account for receptor production after blockade by BAAM. As shown in Fig. 8, data for receptor reappearance after receptor inactivation by BAAM was well described by the model defined in Eq. 3 and the rate of receptor expression obtained independently from cell cycle analysis.

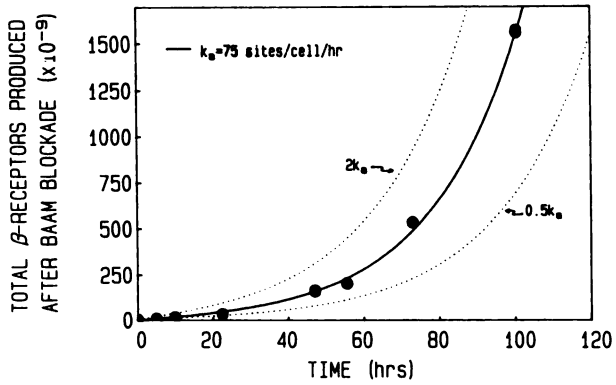


Fig. 8. β-Adrenergic receptor recovery after BAAM blockade and relationship to receptor expression during the S49 cell cycle. Data for cell growth and receptor recovery following receptor inactivation by BAAM (Fig. 7) were combined to determine *total receptor production* (●——●) during the course of receptor reappearance. The *line* through the data was obtained from Eq. 3 (see the text) using the average rate constant of receptor disappearance, k_d = 0.025 hr^{-1} and the rate of receptor expression from cell cycle analysis as an approximation of k_a, the rate of receptor appearance, equal to 75 sites/cell/hr. All other variables were taken from the data shown in Fig. 7.

Discussion

For a number of hormone/neurotransmitter systems, agonist interaction with receptors on target cells alters cellular expression of receptors (12, 25, 28, 29). The key to understanding these agonst-induced changes is a knowledge of how cells regulate the expression of receptors in the absence of agonists, i.e., the "basal state" of receptor metabolism. Various experimental approaches are available that can provide information about basal metabolism of receptors. Although future studies will probably emphasize immunological methods to study receptor turnover, the most common approaches presently available include: 1) the use of inhibitors of protein synthesis to ascertain rates of receptor degradation (24); 2) the heavy isotope-amino acid "density shift" method, which provides estimates of both receptor synthesis and degradation (22, 25); and 3) the use of antagonists capable of binding irreversibly to receptors, thus enabling studies of the kinetics of reappearing receptors (23, 24, 30, 31). Each of these approaches is not without limitations both in its applicability and in the interpretation of experimental results.

Estimates of receptor turnover from the use of inhibitors of protein synthesis cannot exclude the contribution of rapidly turning over cellular proteins that are involved in receptor metabolism. Also, the use of those inhibitors is limited in continuously dividing cells, such as S49 cells, if rates of receptor turnover are slow compared to rates of cell division. Heavy isotopes of amino acids can be useful to study receptor metabolism in postconfluent cell cultures, but problems of cell viability have been encountered in preconfluent cells and in other actively dividing cells (32);[4] cells that have a low quantity of receptor protein also limit the feasibility of this approach. Although irreversible antagonists that block existing receptors can be useful for studies of receptor turnover (e.g., Refs. 23 and 24), care must be taken to show that rates of receptor turnover obtained by this method are not merely a measure of release of antagonist from potentially reutilizable receptor protein. In this study, we combined two independent approaches, irreversible blockade of receptors with BAAM and analysis of receptor expression during the cell cycle, to define β-adrenergic receptor metabolism in S49 cells.

Analysis of the expression of β-adrenergic receptors on S49 cells, separated into cell cycle-specific fractions by centrifugal elutriation, revealed that receptors appear continuously throughout the transition from G_1 to mitosis. This increase in receptor number was proportional to the increase in cell size; thus, receptor density (normalized to cell size) remained constant. These findings are similar to the cell cycle-specific expression of opiate receptors in NG108-15 cells (33). Two

[2] Continuously dividing cells do not reach a steady state level of receptor number *per cell*. As shown in Fig. 3, receptor production is constant and reinitiated in daughter cells after mitosis.

[3] Equations for receptor turnover (Eq. 1) and cell growth (Eq. 2) were combined. The change in receptor number over time (dR/dt) is the net result of receptor appearance minus receptor disappearance, where appearance is expressed as the appearance rate/cell × number of cells/culture. Defining y as R and x as time, the differential equation was solved by Laplace transformation with the Laplace variable, s, yielding (constants described in text):

$$s\hat{R} = \frac{k_a C_o}{s - k_c} - k_d \hat{R} + R_o$$

Solving for R gives

$$\hat{R} = \frac{k_a C_o}{(s - k_c)(s + k_d)} + \frac{R_o}{(s + k_d)}$$

Back transformation yields:

$$R_{(t)} = k_a C_o \left\{\frac{e^{k_c t} - e^{-k_d t}}{k_d + k_c}\right\} + R_o e^{-k_d t}$$

which has been rearranged to Eq. 3.

[4] R. Schwall and G. Erickson, personal communication.

studies of β-adrenergic receptor expression during the cell cycle of rat glioma (C6) cells have been reported (34, 35). Maximum receptor expression (sites/cell) in the former study occurred in late S/G_2 with a dramatic drop during mitosis; G_1 cells contained approximately half the receptor density. The other study reported no change in receptor density (pmol of radioligand bound/mg protein) in S through M phase but a significant increase in G_1. Since, in both studies, synchronization was obtained by double thymidine block, differences in the patterns of expression might be accounted for by the normalization of the data (cells versus mg protein) or by other methodologic differences. At any rate, the results in C6 glioma cells differ from our findings in S49 cells, perhaps because of differences in β-receptor metabolism in cells in monolayer versus suspension culture, because of differences in β_1 (C6 glioma) and β_2 (S49) receptors, or because of intrinsic differences in turnover of membrane receptors in glioma and lymphoma cells.

The blockade of β-adrenergic receptors on S49 cells using BAAM, an antagonist capable of irreversibly alkylating the receptor binding site, proved to be a useful approach for examining the cellular metabolism of β-receptors on S49 cells with minimal perturbation of the cells. Studies with BAAM in asynchronously growing cells indicated that β-receptors have a half-life of approximately 28 hr. Since these cells have a population doubling time of 16–18 hr, receptor protein must be conserved in successive generations. The receptor half-life determined from asynchronous cultures was sufficiently slow so that the initial rate of new receptor expression obtained from synchronized cells (75 sites/cell/hr) could be used as an estimate of the receptor appearance rate, k_a. When data for receptor recovery following irreversible inactivation were analyzed in a model incorporating this estimate for k_a, a good fit for the kinetics of receptor reappearance was obtained. This indicates that values for receptor turnover obtained by inactivation of receptors with BAAM probably provides accurate information regarding the "life cycle" of receptor expression in S49 cells. Although BAAM has been employed for receptor turnover studies *in vivo* (36), this probe has not previously been validated or used in studies of receptor metabolism in cultured cells.

A turnover rate of 28 hr for S49 cell β-adrenergic receptors is considerably slower than the basal turnover rates for several other classes of hormone and neurotransmitter receptors: insulin receptors in 3T3-L1 cells and IM-9 lymphocytes (7.5 and 10.5 hr, respectively; Refs. 22, 29, and 37); EGF receptors in skin fibroblasts and A431 cells (9 and 16 hr, respectively, Ref. 25); and nicotinic cholinergic receptors in BC3H-1 muscle cells (11–16 hr, Ref. 38). However, half-lives as long as 25 hr have been reported for nicotinic cholinergic receptors in developing muscle fibers and cultured chick embryo muscle cells (24, 31) and for α_1-adrenergic receptors on BC3H-1 cells (23).[5] BC3H-1 cells also contain β_2-adrenergic receptors which, upon cell confluence, virtually cease turning over ($t_{1/2} \geq 200$ hr).[5] A half-life for β-adrenergic receptors on human lung (VA2) cells, obtained from the analysis of receptor recovery after blockade with *N*-[2-hydroxy-3-(1-naphthoxy)-propyl]-*N'*-bromoacetylethylenediamine was reported to be 20–30 hr (30); in that study, receptor recovery only reached 60% of control by 35 hr, thus making this estimate of turnover difficult to interpret.

Basal rates of receptor metabolism are strikingly slower than agonist-promoted turnover of β-receptors on S49 cells (12). During incubation of S49 cells with the β-agonist isoproterenol, β-receptors down-regulate with a half-life of 2–4 hr and recover after removal of isoproterenol with a half-life of ~6 hr; recovery is dependent on protein synthesis (12, 39). These agonist-induced alterations in the rates of receptor metabolism represent a severalfold increase over the basal values that we observed in this study for receptor turnover and receptor appearance. Similar agonist-induced accelerations in receptor metabolism have been reported for insulin receptors in 3T3-L1 adipocytes (29) and IM-9 lymphocytes (37), and for EGF receptors in A431 cells and fibroblasts (25). Although agonists alter rates of β-receptor turnover in down-regulated C6 glioma cells (28), receptor recovery after down-regulation apparently does not require protein synthesis, and receptor recycling has been proposed. In another system, 1321N1 human astrocytoma cells, return of β-receptors after agonist-induced down-regulation is dependent on protein synthesis if cells are grown in postconfluent but not in preconfluent cultures (32). It would appear that various target cells can utilize different mechanisms for β-adrenergic receptor metabolism in the absence and presence of agonist.

In summary, a combination of approaches including cell synchronization and receptor inactivation have allowed a preliminary description of the life cycle of β-adrenergic receptors in S49 cells. The receptors appear at a rate of about 75 sites/cell/hr and disappear with a half-life of about 28 hr. These properties of receptor metabolism in cells in the absence of agonist underscore the several-fold enhancement in receptor turnover seen in S49 cells after agonist treatment. Studies with synchronized cells and irreversible blocking agents appear to provide two useful, complementary means to assess basal metabolism of β-adrenergic receptors in cultured cells. The accompanying paper (40) describes the use of these two techniques to assess β-adrenergic response (cAMP generation) in S49 cells.

Acknowledgements

We thank Harvey Motulsky and Vincent Dionne for helpful discussions of this work.

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